

U *com* spectrum of activity of this porcine brain natriuretic peptide, or pBNP, is similar to that of the ,to that of the porcine ANP. A comparison of the amino acid sequence (SEQ ID NOS: 1-3) of a portion of human ANP (hANP) and the pBNP is shown below; the corresponding relevant portion of the porcine ANP is identical to the human sequence.

Please replace the paragraph beginning at page 4, line 6, with the following rewritten paragraph:

- Subsequent papers from this same group at Miyazaki Medical College further characterize these proteins. Sudoh, T., et al., Biochem Biophys Res Comm (1988) 155:726-732, report the isolation of a 32-amino acid natriuretic peptide ("BNP-32") from porcine brain which contains the 26 amino acids of the porcine BNP described above at its C-terminus and an additional N-terminal 6-amino acid extended portion of the sequence (SEQ ID NO: 4) Ser-Pro-Lys-Thr-Met-Arg-. In papers following on subsequent pages, levels of various natriuretic peptides in tissues are reported. Ueda, S., et al., (ibid.), pp. 733-739, utilized a radioimmunoassay to localize and measure the levels of porcine BNP and porcine BNP-32 in the brain and spinal cord. The results showed that both BNP and BNP-32 were major forms of immunoreactive BNP in the porcine brain, and that the highest concentrations were found in the medulla-pons, striatum, and spinal cord. The porcine form of atrial natriuretic peptide (pANP) was also found in the porcine brain but at a level approximately 13 times lower than that characteristic of BNP. Minamino, N., et al. (ibid.), pp. 740-746, report the results of radioimmunoassay for porcine BNP and ANP in peripheral tissue. The concentration of BNP was highest in cardiac atrium of the tissues assayed. The immunoreactive form of this protein was characterized as mostly a 12 kd high molecular weight form; less than 15% of the total immunoreactive BNP in atrial tissue is of the lower molecular weight forms pBNP or pBNP-32.

Please replace the paragraph beginning at page 5, line 35, with the following rewritten paragraph:

- The invention provides the complete gene sequence for pBNP and the prepro form thereof and thus the ability to synthesize large amounts of the proteins encoded by this gene and modified forms thereof. The invention also enables retrieval of the gene sequences encoding proteins of similar amino acid sequence having natriuretic activity from other vertebrate species, and thus provides the ability to synthesize them as well. The cDNA encoding the porcine BNP and its

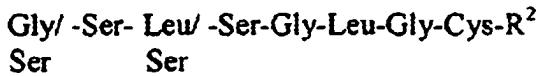
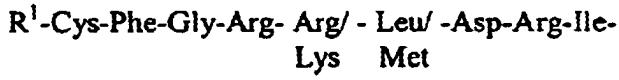
38
precursors and perhaps shorter associated brain proteins is shown in Figure 1A-1D; the segment of this "unprocessed" cDNA which encodes the 26 amino acid pBNP described by Sudoh (supra) is underlined.

Please replace the paragraph beginning at page 6, line 13, with the following rewritten paragraph:

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- Accordingly, in one aspect, the invention is directed to a recombinant cDNA probe containing the sequence encoding the 26-amino acid natriuretic peptide of porcine brain, which comprises the DNA of Figure 1A-1D or an effective portion thereof. The invention is also directed to recombinant DNA sequences retrieved using this probe, or probes derived from it, and thus includes alternatively useful probes which comprise effective portions of the coding sequences for peptides from canine and human sources shown in Figure 5 and 7A-7B.-

Please replace the paragraph beginning at page 6 line 23, with the following rewritten paragraph:

- In another aspect, the invention is directed to peptides having natriuretic activity of the formula (SEQ ID NO: 5):



wherein R^1 (SEQ ID NOS: 6-11) is selected from the group consisting of:-

Please replace the paragraph beginning at page 7 line 25, with the following rewritten paragraph:

- R^2 (SEQ ID NOS: 12-14) is (OH), NH₂, or NR'R" wherein R' and R" are independently lower alkyl (1-4C) or are

11
Asn/
Lys

Asn/ -Val

Lys

Asn/ -Val-Leu

Lys

Asn/ -Val-Leu-Arg

Lys

Asn/ -Val-Leu-Arg- Arg/

Lys Lys

Asn/ -Val-Leu-Arg- Arg/ - Tyr/

Lys Lys His

or the amides (NH₂ or NR'R") thereof,
with the proviso that if formula (1) is

R¹-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Asp-Arg-
Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys-R²

and R¹ is Asp-Ser-Gly-, R² cannot be Asn-Val-Leu-Arg-Arg- Tyr. (SEQ ID NO: 16)

Please replace the paragraph beginning at page 8 line 27, with the following rewritten paragraph:

12 Figure 1A-1D (SEQ ID NOS: 17-30) shows the complete sequence of a retrieved cDNA in unprocessed form which encodes porcine BNP. The portion of the sequence which encodes the 26-amino acid pBNP peptide is underlined and consists of residues 660-723 and 1276-1289 inclusive.

Please replace the paragraph beginning at page 8 line 32, with the following rewritten paragraph:

13 Figure 2 (SEQ ID NOS: 31-37) shows oligonucleotides synthesized as probes for pBNP-encoding cDNA.

Please replace the paragraph beginning at page 8 line 34, with the following rewritten paragraph:

314 Figure 3A-3B (SEQ ID NOS: 38-39) shows the cDNA of Figure 1A-1D with the location of the additional intron established.

Please replace the paragraph beginning at page 9 line 1, with the following rewritten paragraph:

15 Figure 4 (SEQ ID NOS: 40-41) shows the coding portions of the pBNP-encoding cDNA absent the introns.

Please replace the paragraph beginning at page 9 line 4, with the following rewritten paragraph:

16 Figure 5A-5C (SEQ ID NOS 42-43) shows the DNA and deduced protein sequence for the coding portions of the gene encoding a canine protein with natriuretic activity.

Please replace the paragraph beginning at page 9 line 10, with the following rewritten paragraph:

17 Figure 7A-7B (SEQ ID NOS: 44-45) shows the DNA and deduced amino acid sequence of the human genomic clone encoding the human NRP.

Please replace the paragraph beginning at page 9 line 13, with the following rewritten paragraph:

18 Figure 8 (SEQ ID NOS: 46-48) shows a comparison of the amino acid sequences of the prepro forms of the porcine, canine and human proteins of the invention.

Please replace the paragraph beginning at page 9 line 21, with the following rewritten paragraph:

[As used herein, "brain natriuretic peptide (BNP)" refers to an amino acid sequence which is encoded by a DNA capable of hybridizing to an effective portion of the DNA shown in Figure 1A-1D under defined stringency conditions, and which has natriuretic activity. It is believed that the brains of all vertebrates contain a subpopulation of peptides with this activity which comprise peptides analogous to that disclosed herein as pBNP and longer precursor proteins containing this amino acid sequence, as well as active fragments thereof.]

Please replace the paragraph beginning at page 9 line 30, with the following rewritten paragraph:

[As used herein, "porcine brain natriuretic peptide (pBNP)" refers to the 26 amino acid sequence isolated by Sudoh *et al.*, and set forth hereinabove. "pBNP-encoding cDNA". refers to the nucleotide sequence shown in Figure 1A-1D herein, comprising residues 660-723 and 1276-1289 inclusive. The separation in the cDNA of the pBNP codons is presumably due to incomplete processing of the mRNA which formed the template for this particular clone. This clone was deposited at the American Type Culture Collection, Rockville, MD, on 10 June 1988 and has accession number ATCC 40465.]

Please replace the paragraph beginning at page 10 line 6, with the following rewritten paragraph:

[The pBNP-encoding cDNA shown in Figure 1A-1D, because it contains additional sequences encoding precursor proteins, and, as explained below, presumably contains nucleotides corresponding to an additional intron besides that represented by the sequence separating the pBNP-encoding portion per se, can be used as an effective probe to obtain either genomic or cDNA sequences encoding corresponding associated brain natriuretic peptides in various vertebrate species. "Precursor brain natriuretic peptide" as used in the present application refers to peptides with natriuretic activity encoded by the gene sequence from which, for example, the pBNP protein is derived but processed so as to obtain peptides of different length. Similar processing differences

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Additional terminology which is useful is the term "prepro" NP, which refers to the encoded peptide having both the native associated signal sequence which effects secretion of the various forms of the peptide with natriuretic activity and an amino acid sequence of the secreted peptide which is fused upstream of the cyclic portion absolutely required for this activity. The "pro" form having the upstream sequence may represent the circulating form of the peptide. With respect to the three specific embodiments included within the present invention, which are shown in detail in Figures 3A-3B, 5A-5C, and 7A-7B for porcine, canine and human proteins, respectively, the location of the putative signal sequences representing the "pre" sequence is shown in each figure, as well as the full-length mature protein, which is thought to be a precursor form designated the "pro" form. Because various processing sites are available, as indicated by the upward-pointing arrows in these figures and in the composite sequences shown in Figure 8, attempts to make a fine-line and definite distinction between the "pro" NP and "NP" are probably meaningless. The peptides defined by the invention are set forth in formula (1) above and have natriuretic activity, regardless of the length of the N-terminal form preceding the identified 26-amino acid regions corresponding to the porcine "BNP" of Sudoh, or attached to the cyclic portion thereof.

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Please replace the paragraph beginning at page 12 line 33, with the following rewritten paragraph:

The invention, in one aspect, is directed to all members of the group of porcine BNP proteins encoded in the cDNA shown in Figure 1A-1D, and to conservative modifications thereof. The deduction of the amino acid sequence encoding the longest precursor protein, and therefore deduction of the processed forms, can be accomplished using the unprocessed cDNA here provided. In this standard approach, oligonucleotide sequences representing short portions of the cDNA spanning the potential intron--i.e., between residues 100 to about 660--are synthesized, labeled, and used to probe Northern blots of mRNA isolated from cells producing BNP. Most mRNAs will be in processed form; hence, those oligonucleotides which successfully hybridize to the proper length message represent coding regions of the cDNA. Those which do not readily hybridize represent intron regions. By using overlapping synthetic cDNAs, the intron position can be precisely

21 cont'd

presumably exist in other vertebrates as well; the entire class of such natriuretic peptides is retrieved by the DNA probe of the invention. For example, examination of the reading frame of the pBNP-encoding DNA shows an N-terminal extension so that N-terminally extended peptides can be postulated. It has been shown that the ANP precursor "pro-ANP" is processed differently in atrial and brain tissues leading to different ANP-peptides. By analogy to the peptides found in the atrium, it is postulated that an important peripheral form of BNP would be the 29-residue peptide of pBNP N-terminally extended with the tripeptide Thr-Met-Arg. Further N-terminal extended peptides with the additional upstream residues Ser-Pro-Lys and Gly-Ile-Arg-Ser-Pro-Lys are also expected. Thus, examination of the reading frame which contains the pBNP also permits postulation of additional upstream processing sites which would extend the N-terminal sequence further.

Please replace the paragraph beginning at page 11 line 1, with the following rewritten paragraph:

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- Other extended precursor peptides are discoverable through standard techniques using the sequence information of Figure 1A-1D. It is clear, by analogy with atrial natriuretic peptide precursors, that the start of the longest precursor, perhaps including a signal sequence, is at the methionine codon shown in the uppermost reading frame in the line spanning nucleotide 61 and 120, or at the closely positioned downstream ATG. Therefore, it is clear that the reading frame is not maintained from this start of translation into the pBNP encoding region. This indicates that there is-at least one other intron transcribed into the cDNA clone retrieved. The location of this intron and deduction of the full sequence for the longest form of precursor peptide is described in further detail below. In any event, precursor BNP peptides associated with pBNP include other natriuretic peptides encoded by this depicted gene; analogous groups of peptides are collectively designated natriuretic "NP" peptides in other species.

Please replace the paragraph beginning at page 11 line 21, with the following rewritten paragraph:

B24
identified. This permits deduction of the complete sequence encoding the largest precursor protein, and defines the sequence from which the associated BNP proteins are formed.

Please replace the paragraph beginning at page 13 line 18, with the following rewritten paragraph:

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-In a modification of this approach, partial cDNA fragments were generated in amplified form from mRNA isolated from porcine atrium. The cDNA for amplification was obtained by hybridization of poly A⁺ RNA isolated from this tissue with the oligonucleotide 3895. Amplification was performed using a polymerase chain reaction wherein the oligonucleotide primers corresponded to bases 100-123 (identity strand) and 652-685 (complementary strand) as shown in Figure 1A-1D. Two bands are obtained when the amplified products are analyzed on preparative agarose gels; the larger band in low relative abundance presumably represents the smaller DNA derived from the unspliced precursor, and the more prominent band is assumed to be the more fully processed cDNA. When this band was eluted from the gel and sequenced, the stretch corresponding to bases 223-468 of Figure 1A-1D was not present, and the recovered DNA had the sequence shown in Figure 3A-3B.]

Please replace the paragraph beginning at page 13 line 35, with the following rewritten paragraph:

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-Thus, using standard techniques, the location of the putative upstream intron, which would correspond to that found in atrial natriuretic peptide precursors, as described by Greenberg et al., Nature (1984) 312:656-658 was easily obtained. As shown in Figure 4, which represents solely the portions shown as coding sequences of Figure 3A-3B, a reading frame of 131 amino acids is obtained. It is believed that the signal sequence is represented by amino acids 1-25, and that the cleavage site converting the prepro form of porcine BNP to the pro form is between Ser₂₅ and His₂₆ at position 26 of the prep sequence, as shown in Figure 4. The sequence of the porcine BNP reported by Sudoh having 26 amino acids is represented by amino acid residues 81-106 (106-131).

Please replace the paragraph beginning at page 14 line 14, with the following rewritten paragraph:

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-In addition to providing access to the class of porcine BNPs encoded on the retrieved cDNA, the cDNA of Figure 1A-1D provides access to the corresponding precursor gene encoding the class of associated NRP proteins from various vertebrate species.-

Please replace the paragraph beginning at page 15 line 32, with the following rewritten paragraph:

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-In practice, the porcine DNA of Figure 1A-1D was able to retrieve genes encoding related proteins in genomic libraries from a variety of other species, either directly or indirectly. Genomic libraries from pig, rat, dog, cat and rabbit showed the ability to hybridize to the probe of Figure 1A-1D under at least one of the conditions:-

Please replace the paragraph beginning at page 16 line 10, with the following rewritten paragraph:

29
-Human genomic DNA did not hybridize to the DNA sequence of Figure 1A-1D under these conditions, but could be obtained indirectly using the other mammalian DNAs obtained using this probe.-

Please replace the paragraph beginning at page 16 line 14, with the following rewritten paragraph:

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-By obtaining the canine DNA through use of the porcine probe, an insert having the sequence shown in Figure 5 was obtained, was designated pdBNP-1, and was deposited at the American Type Culture Collection on 14 December 1988 under Accession No. ATCC-67862.

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Using this sequence as a probe, a clone obtained from EcoRI-digested human genomic DNA was found which encodes a similar protein having natriuretic activity. This human DNA has the sequence shown in Figure 7A-7B, was designated phBNP-1, and was deposited at the American Type Culture Collection on 14 December 1988 under Accession No. ATCC-67863.

Please replace the paragraph beginning at page 16 line 25, with the following rewritten paragraph:

331
The amino acid sequence encoding the putative prepro forms of peptides with natriuretic activity from porcine, canine, and human species are shown in Figure 8. It is apparent that the porcine and canine species are more homologous in the region putatively responsible for natriuretic activity than the human sequence. Using the information in Figure 8, a class of peptides having natriuretic activity can be defined. This class is of the formula (SEQ ID NO 5):

Please replace the paragraph beginning at page 17 line 1, with the following rewritten paragraph:

332
-R¹-Cys-Phe-Gly-Arg- Arg/ -Leu/ -Asp-Arg-Ile-
Lys Met

Gly/ -Ser- Leu/ -Ser-Gly-Leu-Gly-Cys-R²
Ser Ser

wherein R¹ (SEQ ID NOS: 6-11) is selected from the group consisting of:

333
Please replace the paragraph beginning at page 18 line 1, with the following rewritten paragraph:

R² (SEQ ID NOS: 12-14) is (OH), NH₂, or NR'R" wherein R' and R" are independently lower alkyl (1-4C) or is

335
Asn/
Lys

Asn/ -Val
Lys

Asn/ -Val-Leu

Lys

Asn/ -Val-Leu-Arg

Lys

Asn/ -Val-Leu-Arg- Arg/

Lys Lys

Asn/ -Val-Leu-Arg- Arg/ - Tyr/

Lys Lys His

36

or the amides (NH_2 or $\text{NR}'\text{R}''$) thereof,

with the proviso that if formula (1) (SEQ ID NO: 15) is

$\text{R}^1\text{-Cys-Phe-Gly-Arg-Arg-Leu-Asp-Asp-Arg-}$
 $\text{Ile-Gly-Ser-Leu-Ser-Gly-Leu-Gly-Cys-R}^2$

and R^1 is Asp-Ser-Gly-, R^2 cannot be Asn-Val-Leu-Arg-Arg- Tyr (SEQ ID NO: 16).
37

Please replace the paragraph beginning at page 19 line 22, with the following rewritten paragraph:

A deduced (or otherwise generated) peptide sequence falls within the scope of certain natriuretic proteins of the invention, provided that the DNA encoding it directly or indirectly hybridizes to the pBNP-encoding cDNA of Figure 1A-1D under conditions corresponding to the stringency represented by hybridization in buffer containing 20% formamide, 5 x Denhardt's, 6 x SSC, 100 mg/ml RNA, and 0.05% sodium pyrophosphate at 42°C, followed by washing at 60°C at 1 x SSC, 0.1% SDS, or under conditions (1) or (2) described above. In addition, the peptide encoded by this DNA must exhibit natriuretic activity assayed as described below.
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Please replace the paragraph beginning at page 19 line 34, with the following rewritten paragraph:

By "direct hybridization" is meant that the DNA hybridizes to an DNA which is capable itself of hybridizing to the porcine BNP of Figure 1A-1D. Thus, the human sequence shown in
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B37
5C. []
Figure 7A-7B indirectly hybridizes to the porcine BNP through the canine sequence of Figure 5A-

Please replace the paragraph beginning at page 20 line 7, with the following rewritten paragraph:

B38
The invention is also directed to modified forms of the BNP proteins encoded by the cDNA of Figure 1A-1D. One or two of the positions of these BNPs can be altered, so long as activity is retained. Conservative amino acid substitutions are preferred--that is, for example, aspartic/glutamic as acidic amino acids; lysine/arginine/histidine as basic amino acids; leucine/ isoleucine, methionine/valine as hydrophobic amino acids; serine/glycine/alanine/threonine as hydrophilic amino acids. However, as the peptides need not be prepared by recombinant methods or from the gene, the substitutions may include nonencoded amino acids such as the D- or beta- amino forms.

B39
Please replace the paragraph beginning at page 28 line 4, with the following rewritten paragraph:

The foregoing methods to synthesize the BNP of the invention are not intended to be limiting, and the BNP of the invention may be prepared in any convenient manner. The BNP is required only to be encoded by a gene which hybridizes under the above-specified stringent conditions to the cDNA of Figure 1A-1D and to show natriuretic activity in the receptor assay described below.

Please replace the paragraph beginning at page 38 line 16, with the following rewritten paragraph:

B40
The DNA sequence of the insert from clone 14 is shown in Figure 1A-1D. The coding region for BNP is present within the clone; however, it is interrupted by what appears to be an

B⁴⁰
intron at residue Val₂₂ of the 26-amino acid BNP. Therefore, it appears that this clone contains an unprocessed mRNA with one or more introns present.

Please replace the paragraph beginning at page 38 line 33, with the following rewritten paragraph:

- Poly A⁺ RNAs were isolated from porcine atrial tissue using the guanidinium isothiocyanate method of Chirgwin, J.M., Biochemistry (1979) 18:5294-5299, followed by oligo-dT cellulose chromatography. Approximately 2 ug of the porcine atrial mRNA was incubated with 400 ng of oligonucleotide 3895 (supra) as primer in a 20 ul reaction containing 0.5 mM dNTPs, 50 mM Tris-HCl, pH 8.3, 10 mM magnesium chloride, 10 units of RNasin, and 50 units of reverse transcriptase. Subsequent amplification of the resulting DNA was performed as described by Saiki, R.K., Science (1988) 239:487-491. After incubation for 1 hr at 37°C, half of the reaction was diluted to 100 ul in 67 mM Tris-HCL, pH 8.8, 6.7 mM magnesium chloride, 16.6 mM ammonium sulfate, 10 mM mercaptoethanol, 6.7 uM EDTA, 1 mM dNTPs, 10% DMSO, and 400 ng of each primer oligonucleotide. The oligonucleotide primers were those corresponding to bases 100-123 (identity strand) and 652-685 (complementary strand) of the pBNP clone shown in Figure 1A-1D.

Please replace the paragraph beginning at page 40 line 35, with the following rewritten paragraph:

B⁴²
- Genomic DNA from pig, rat, dog, cat, rabbit and human organisms were probed on Southern blots using the cDNA illustrated in Figure 1A-1D herein under two different hybridization conditions:

Please replace the paragraph beginning at page 41 line 10, with the following rewritten paragraph:

343
-A dog genomic library obtained from Clontech Inc. yield 2 clones under the condition (1) above and the DNA from these identified clones was digested with HaeIII or AluI and subcloned into M13. The resulting plaques were screened for hybridization to the porcine probe, and positive clones were sequenced. The identity of the clone was confirmed by detection of the BNP-encoding sequence of Figure 1, and the 2.9 kb HindIII fragment containing the entire gene was then subcloned into pBR322, and designated pdBNP-1. The DNA sequence of the portion of this clone encoding the BNP gene is shown in Figure 5A-5C, and the pdBNP-1 plasmid was deposited at the American Type Culture Collection on 14 December 1988 and has Accession No. ATCC-67862.

Please replace the paragraph beginning at page 41 line 23, with the following rewritten paragraph:

344
-Although a human genomic library failed to yield signals corresponding to hybridization with the probe using the porcine DNA of Figure 1A-1D, use of pdBNP-1 as a probe under condition (1) above produced several distinct bands that could be visualized in blots of digested human genomic DNA, as shown in Figure 6. A preparative agarose gel was utilized to isolate EcoRI-digested human genomic DNA in the 6-7 kb size range, which isolated DNA was then cloned into lambda-ZAP2 (Strategene Inc.), packaged, and the resulting mini-library was screened using the hybridization condition (1) above. Seven positive signals were purified and the insert subcloned into pBLUSCRIPT vector. The sequences of the M13 subclones of hybridization-positive HaeIII and AluI-digested plasmid DNA were determined. The sequence of the coding region of the plasmid, phBNP-1, is shown in Figure 7A-7B and the plasmid was deposited at the American Type Culture Collection on 14 December 1988 with Accession No. ATCC-67863.

ABSTRACT

A separate page with the abstract is enclosed.